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TITLE OF THE INVENTION

THE STAPHYLOCOCCUS AUREUS FIBRINOGEN-BINDING PROTEIN Efb (SAC3) BINDS C3 AND INHIBITS COMPLEMENT ACTIVATION

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Title: The *Staphylococcus aureus* Fibrinogen-binding protein Efb (SAC3) Binds C3 and Inhibits Complement Activation

Abbreviations footnote: SA, *S. aureus*; SAC3, *S. aureus* C3-binding protein; MIM, microbial immunomodulatory molecule

Introduction

Staphylococcus aureus (SA) is typically an innocuous commensal organism that can colonize the skin and anterior nares of 20% of humans without causing disease or discernable clinical symptoms (1, 2). However, SA remains one of the world's primary health threats and the emergence of antibiotic-resistant SA strains pose severe health threats in hospitals and communities (1, 3). Furthermore, the broad spectrum of diseases that can result from SA infections (e.g. skin infections, arthritis, or septic shock) is a reflection of this organisms capacity to not only colonize a variety of different tissues via different MSCRAMM (Microbial surface Components Recognizing Adhesive Matrix Molecules)-ligand interactions (4-14) but to also circumvent a variety of immune surveillance systems which result in the persistence of SA in radically different environments within the host organism (15-19).

Because tissue colonization is a critical first step in any infection process and pathogens associated with persistent infections must constantly avoid host immunity to survive, it is not surprising that some MSCRAMMs can also serve as MIMs (Microbial Immunomodualtory Molecules) (18). For example, the secreted SA Map (MIIC Analog Protein) not only adheres to a variety of extracellualr matrix (ECM) components (20, 21) but can also negatively affect T cell immunity and prevent neutrophil migration (15, 18) and the Streptococcal M protein which binds fibrinogen (Fgn) can also interfere with complement activation (alternative pathway) by mechanisms not clearly defined (22).

The complement system represents the most 'primitive' line of defense against infectious agents. Complement systems exist in all vertebrates (23) and complement-component analogs can be found in some nonvertebrates (e.g. horseshoe crab and insects) (23). Evolutionarily, the complement system at many levels became associated with humoral immunity and functions as a primary antibody effector mechanism (23). The importance of the complement system as a primary and first line of defense mechanism in vertebrates is mirrored by the fact that infectious agents including bacteria, viruses, protazoans, helminths, and fungi, have developed survival strategies which interfere with, avoid, or manipulate complement system components (23-26). Although the complement system is comprised of at least 20 immunologically and chemically distinct proteins, the effector functions of the complement cascade are carried out by the components designated C1-C9 (27). A critical component of the complement cascades is C3 which is the common link between the alternative, classical and mannose binding lectin (MBL) pathways of complement activation (27).

In a mouse model of SA-induced septicaemia and septic arthritis, complement-depleted (with cobra venom factor) mice presented with significantly increased disease severity, suggesting a role for complement in controlling haematogenously-acquired SA infections (28). Conversely, intradermally-infected, C3-deficient mice were more resistant to SA infections compared to wild-type control mice, suggesting the possibility that SA may actively interact with C3 (Haviland and Wetsel, unpublished observations) (29, 30). Since no SA immunoregulatory proteins with complement binding activity had been described to date, we examined the possibility that SA may generate a C3-binding protein.

We have identified a 19 kDa protein secreted by SA that can bind to the complement protein C3 and have designated this protein SAC3 (*S. aureus* C3-binding protein). N-terminal sequencing of SAC3 identified this protein as the SA extracellular fibrinogen-binding protein (Efb) (31-33). Efb is a constitutively secreted protein that not only binds Fgn, but can interfere with platelet aggregation and is hypothesized to play a role in delaying wound healing (34). The data presented in this report suggest that SAC3 is a MIM that may be involved in SA survival and persistence.

Materials and Methods

Identification of SAC3. *Escherichia coli* strain JM101, *Staphylococcus carnosus* strain TM300, and *Staphylococcus aureus* strain Newman were grown under shaking conditions overnight at 37°C in 5 ml Lennox broth (Sigma-Aldrich, St. Louis, MO, USA) as described previously (18, 35). Bacteria were washed in phosphate buffered saline (PBS, pH 7.4) and total protein was quantified using the bicinchoninic acid protein assay (BCA) (Pierce Chemical Co., Rockford, IL, USA). Bacteria (20 μg) or 20 μl of SA supernatant from an overnight culture were fractionated by SDS-PAGE (12% gel) under reducing conditions and examined by staining with 0.05% Coomassie brilliant blue R250 or subjected to electro-transfer to a 0.45 μm Immobilon-P™ PVDF (polyvinylidene fluoride) membrane (Millipore, Bedford, MA, USA) as described previously (35). Western-ligand blots were performed by blocking the membranes overnight in 5% non-fat dry milk in TBST (0.15 M NaCl, 20 mM Tris-HCL, 0.05% Tween 20 (Sigma-Aldrich), pH 7.4) overnight at 4°C and probed with 5 μg of digoxigenin-labeled C3 or C3b (Advanced Research Technologies, San Diego, CA, USA). Binding was visualized by incubating with a 1:15,000 dilution of anti-digoxigenin-alkaline phosphatse (AP) conjugated Fab fragments (Roche Diagnostics, Mannheim, Germany) and developed with 10 ml of 1-Step™ NBT/BCIP solution (Pierce). All incubations were performed in 15 ml of 1% TBST for 1 h with shaking at room temperature and membranes were washed 3 times (5 min with shaking) in TBST between all steps. C3 and C3b were digoxigenin-labeled as described previously according to manufacturer's instructions (36).

Further characterization of the ~19 kDa C3-binding protein was performed by fractionating 50 µl of supernatant from an overnight SA culture on a large (20 cm) 15% SDS-PAGE gel and transferring onto a PVDF membrane as described above. The membrane was subsequently stained with 0.05% Coomassie brilliant blue R250 solution for 20 min and destained with 50% methanol. Two candidate bands of interest were noted on the membrane and sent to the Protein Chemistry Laboratory (Texas A&M University, College Station, TX, USA) for N-terminal sequencing which resulted in the identification of residues S-E-G-Y-P-R-E-K-K and F-T-F-E-P-F-P-T-N-E corresponding to the Efb (PubMed accession number Q08691) and the SA1755 (PubMed accession number E89983) protein sequences from SA, respectively.

Cloning of the *sac3* and *sa1755* genes from *S. aureus* strain Newman. The *sac3* and the *sa1755* genes excluding the 5' signal sequence were amplified by polymerase chain reaction (PCR) using *S. aureus* strain Newman DNA as a template. The following oligonucleotide primers were used: 5'-CGC GGA TCC CCA AGA GAA AAG AAA CCA GTG AGT A-3' forward primer and 5'-AAC TGC AGA GTT TTA TTT AAC TAA TCC TTG-3' reverse primer and 5'-CGC GGA TCC CCG TTT CCT ACA AAT GAA GAA-3' forward primer and 5'-AAC TGC AGC TAG TAT GCA TAT TCA TTA-3' reverse primer for *sac3* and *sa1755*, respectively (IDT Inc, Coralville, IA, USA). *Bam HI* and *Pst I* restriction enzyme sites (underlined) were incorporated into the forward and reverse primers, respectively. Each reaction contained 500 ng of template DNA, 5 pmol of forward and reverse primers, 25 mM dNTPs, 1.5 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.01% Tween-20, and 2 units of *Taq* DNA polymerase (CLP, San

Diego, CA, USA). The reaction was performed on a Perkin-Elmer DNA Thermocycler using the following conditions: 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min for 30 cycles. The resulting PCR amplifications resulted in 400 or 351 base pair products that were subsequently TA-cloned into the pCRT7/NT-TOPO expression vector (Invitrogen, Carlsbad, CA, USA) and designated pCRT7/NT-SAC3 or pCRT7/NT-SA1755, respectively. Nucleotide sequencing was performed with the Sequenase version 2.0 sequencing kit (US Biochemicals) according to the manufacturer's instructions and by automated sequencing (Molecular Genetics Core Facility in the Department of Microbiology and Molecular Genetics, University of Texas-Houston Medical School). Sequencing of *sac3* and *sa1755* was performed using the oligonucleotide primers T7 forward 5'-TAA TAC GAC TCA CTA TAG GG-3' and T7 reverse 5'-CTA GTT ATT GCT CAG CGG TGG -3' (IDT Inc).

Expression and Purification of Recombinant Proteins. The SA proteins SAC3, SA1755, Map19 (18) and the *Staphylococcus epidermidis* Fgn-binding protein (SdrG) (29) were expressed as recombinant N-terminal His-tagged proteins that allowed for purification using metal ion-chelating chromotography as described previously (18, 36). Map19 and SdrG were expressed using the pQE expression vector (QIAGEN Inc., Chatsworth, CA, USA) in *E. coli* (JM101) (Stratagene, La Jolla, CA, USA) and SAC3 and SA1755 were expressed using the pCRT7/NT-TOPO (Invitrogen) expression vector in *E. coli* (BL21) harboring the corresponding plasmids, respectively. Map19 is a secreted SA protein that can be detected in SA supernatants (18) and was used as negative control protein for various assays.

E. coli were grown at 37°C in Lennox broth (LB) containing the appropriate antibiotics until they reached an A_{600} of 0.6 (37). Isopropyl-β-D-thiogalactopyranoside (IPTG) (Life Technologies) was added to a final concentration of 0.2 mM, and the cells were incubated at 37°C for an additional 4 h. Cells from a 1 L culture were harvested by centrifugation and resuspended in 10 ml "binding buffer" (BB) (20 mM Tris HCl, 0.5 M NaCl, 15 mM imidazole, pH 8.0) and lysed in a French pressure cell at 11,000 pounds/inch^2 (36). The lysate was centrifuged at 40,000 x g for 15 min and the supernatant filtered through a 0.45 μm filter. A 1 ml iminodiacetic acid Sepharose column (Sigma, St. Louis, MO) was charged with 75 mM $NiCl_2 \cdot H_2O$ and equilibrated with BB. The filtered supernatant was applied to the column and washed with 10 volumes of BB, then 10 volumes of BB containing 60 mM imidazole. The bound proteins were eluted with BB containing 200 mM imidazole, dialyzed against PBS containing 10 mM EDTA, then dialyzed against PBS (36). Protein concentrations were determined by BCA (Pierce) and proteins were stored at -20°C until use.

Western-Ligand Blot Analysis. Recombinant proteins (4 μg each), C3b or C3 (2 μg), human Fgn (2 μg) (29), or human serum (20 μl of a 1:20 dilution in PBS) (Diamedix, Miami, FL, USA) were subjected to SDS-PAGE under reducing conditions and visualized with Coomassie brilliant blue or transferred onto a supporting membrane and blocked overnight as described above. All incubations and washes were performed as described above.

Recombinant proteins were probed with either 1 μg or 7.5 μg of digoxigenin-labeled C3b, C3 or Fgn, respectively, prior to incubation with anti-digoxigenin-AP-labeled Fab fragments. Blots were developed as described above.

C3b, Fgn, or human serum were probed with either 80 μg SAC3 (or control proteins) or a 1:2000 dilution of biotin-labeled polyclonal chicken anti-human C3 antibodies. The secondary incubation for recombinant protein-probed blots consisted of a 1:5000 dilution of a monoclonal mouse (IgG2a) anti His antibodies (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) followed by a goat anti-mouse AP-labeled secondary antibody (ICN/Cappel Biomedicals Inc., Aurora, OH, USA). The secondary incubation for chicken anti-C3-probed blots consisted of a 1:10,000 dilution of avidin-AP (ICN/Cappel). Blots were developed as described above.

Direct Binding ELISA. Immulon-1 microtiter plate wells (Dynatech Laboratories, Chantilly, VA, USA) were coated with either 0.25 μg C3b or Fgn in 50 μl of PBS for 2 h. The plates were washed and then blocked with 200 μl of Super Block (Pierce) for 1 h. After washing, recombinant proteins (0-20 μM in 100 μl final volume/well) were added to corresponding wells and incubated for 1 h. After washing, 100 μl of a 1:5000 dilution of monoclonal mouse anti-His antibodies (Amersham Pharmacia Biotich, Inc.) were added to the wells and incubated for 1 h. After washing, 100 μl of a 1:5000 dilution of goat anti-mouse AP-conjugated antibodies (ICN/Cappel) was added to corresponding wells and incubated for 1 h. After washing, 100 μl of a 1 mg/ml Sigma 104 phosphatase substrate (Sigma) dissolved in 1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8 was added and allowed to develop for 1 h. Plates were read at 405 nm using a microplate reader

(Molecular Devices, Menlo Park, CA, USA). Plates were washed 5 times between all steps with PBS-0.05% Tween 20 and all incubations took place at 37°C. All dilutions were made using Super Block unless otherwise specified.

Complement Activity Assays. The effect of SAC3 on both the classical and alternative pathways was examined. Total serum complement assays or CH50 is the traditional method for determining functional complement activity. A clinical diagnostic kit, EZ Complement CH50 assay kit, (Diamedix) was used to evaluate the effects of recombinant SAC3 on classical complement pathway activation. A 20 μM (20 μl) concentration of either SAC3, or SdrG were incubated with 20 μl of the standard reference serum (Diamedix) at 37°C for 1 h. 20 μl of each of the standard, high and low reference serum samples were incubated with 20 μl of PBS and incubated in the same fashion as test serum samples. During this incubation, tubes containing a standardized suspension of sheep erythrocytes (sRBC) coated with anti-erythrocyte antibodies were allowed to equilibrate to room temperature. After this incubation, 10 μl of the serum/protein mix or control serum samples were added to individual sRBC-containing tubes and incubated at room temperature for 1 h. The tubes were centrifuged at 1800 rpm for 10 min and the absorbance of the supernatants was read at 405 nm to determine the percent of erythrocyte cell lysis. The data are expressed as percent lysis of the standard reference serum.

Alternative pathway activation was measured using a procedure adapted from Advance Research Technologies. Briefly, the recombinant proteins SAC3 or SdrG (10 μl each at a concentration of 20 μM) were incubated with 10 μl of standard reference serum

(Diamedix) at 37°C for 1 h. To each recombinant protein/serum mixture, 10 µl of 100 mM $MgCl_2$/EGTA, 10 µl of GVB (gelatin-veronal buffer) (Advanced Research Technologies) (38), and 30 µl of a 5 x 10^8/ml rabbit erythrocytes (Advanced Research Technologies) stock were added and incubated at 37°C for 30 min. Reactions were stopped by adding 1 ml of ice-cold GVB. The tubes were centrifuged at 1800 rpm for 10 min and the absorbance of the supernatants was read at 405 nm to determine the percent of erythrocyte cell lysis. The data are expressed as percent lysis of the complement standard reference serum.

Results

***S. aureus* Secretes A C3-Binding Protein.** To determine if SA expressed any C3-binding proteins, whole cell lysates from *E. coli*, *S. carnosus*, *S. aureus* or *S. aureus*-supernatants were subjected to SDS-PAGE and stained with Coomassie brilliant blue (Fig. 1*a*) or transferred onto a PVDF membrane and probed with digoxigenin-labeled C3b (Fig. 1*b*). An approximately 19 kDa band specific for C3b was detected in both SA whole cell lysates and SA supernatants but not in control cell lysates (Fig. 1*b*). C3b, which contains a hydrolyzed thioester bond, and not C3 was used as a probe to demonstrate that binding to bacterial proteins was independent of the thioester conformation. However, blots probed with digoxigenin-labeled C3 revealed similar staining and blots probed with an AP-labeled secondary antibody alone revealed no nonspecific binding (data not shown). All subsequent assays were performed with C3b or digoxigenin-labeled C3b as a precautionary measure against nonspecific binding.

Efb (SAC3) Is The *S. aureus* C3-Binding Protein. Two candidate proteins with an approximate size of 19 kDa as depicted in Fig 1*b* were selected for N-terminal sequencing from Coomassie-stained PVDF membranes. These proteins were identified as the ~19 kDa and ~17 kDa Efb (33) and SA1755 proteins, respectively. Efb will be referred to in this report as SAC3. Recombinant His-tagged forms of SAC3 and SA1755 were cloned and expressed (Fig. 2*a*) and the capacity of these proteins to bind C3b and Fgn was assessed by Western-ligand blot analysis (Fig. 2*b-c*). Recombinant forms of the secreted SA protein Map19 and the *S. epidermidis* Fgn-binding protein (SdrG) were used

as controls (18, 29). Only SAC3 bound digoxigenin-labeled C3b and both SAC3 and SdrG, but neither SA1755 nor Map19, bound to digoxigenin-labeled Fgn (Fig. 2*b* and 2*c*, respectively).

When C3b, Fgn, and human serum were subjected to SDS-PAGE (Fig. 3*a*, lanes 1-3, respectively), transferred onto to PVDF membranes and probed with either recombinant SAC3 or with anti-human C3 antibodies (Fig. 3*b* and 3*c*, respectively) distinct binding to the C3 α–chain was observed (Fig. 3*b* and 3*c*, lanes 1 and 3). The minor shift in molecular weight observed between the C3b α-chain and the C3 α-chain detected in human serum (Fig 3*b* and 3*c*, lanes 1 and 3, respectively) is due to the proteolytic cleavage of the 77-residue peptide (C3a) removed from the amino terminus of the C3 α-chain that results in the formation of C3b.

Confirmation that SAC3 was binding specifically to C3 in the human serum sample was obtained by probing a corresponding blot with anti-human C3 antibodies (Fig. 3*c*). Blots probed with either SAC3 or anti-human C3 antibodies revealed binding to bands of identical molecular weights, suggesting that in human serum SAC3 only bound C3. Blots probed with SA1755 or with secondary antibody alone revealed no cross-reactive binding (data not shown). SAC3-Fgn binding was less conspicuous and only very weak binding to the β and γ chains was detected (Fig. 3*b*, lane 2). The weak Fgn binding by SAC3 is somewhat contradictory to previous reports that suggested a strong binding to the Fgn α-chain (34, 39).

Further binding analysis revealed a dose-dependent and saturable binding of SAC3 to both C3b- or Fgn-coated microtiter wells compared to SdrG that bound only to Fgn-coated wells (Fig. 4*a-b*). Neither SdrG (Fig. 4*a*) nor SA1755 (data not shown)

bound to C3b-coated wells. One possibility for the differences in SAC3 binding to Fgn between Western-ligand blots and by ELISA may be due to a different conformational status of the Fgn in the two assays. In the Western-ligand blots, the Fgn may be at least partly denatured (following SDS-PAGE) when the bacterial protein encounters Fgn, whereas a properly folded form of Fgn may be the target of SAC3 binding in the ELISA-type assay.

SAC3 Interferes With Alternative And Classical Pathways. Although direct binding between SAC3 and C3 had been established, it remained unclear what function if any SAC3 served with regard to complement activation. Because C3 is a key initiating protein for the alternative, classical and MBL complement pathways, C3 inactivation would essentially eliminate all complement activity and provide a potent immunomodulatory component for SA. Functional assays for both the classical and alternative pathways were used to assess the capacity of SAC3 to interfere with complement activity. Complement-mediated lysis of red blood cells by either the classical or alternative pathways was inhibited if human serum was preincubated with SAC3 but not with control proteins (Fig. 5*a* and 5*b*, respectively), suggesting that SAC3 can function as a complement inhibitory protein by binding to C3. SA1755 had no complement function inhibitory properties (data not shown).

Discussion

The broad spectrum of diseases which can result from SA infections is a function of various parameters: the bacterium's versatility allowing for the colonization of various tissues and avoidance of host immunity, the genetic make up and immune status of the host, and the portal of entry (e.g. intravenous, cutaneous, or intraperitoneal) (1, 18, 40, 41). Complement is an essential component of the innate immune system and in higher vertebrates and mammals increased bacterial and viral infections are concomitant with complement protein deficiencies (23). It is therefore not surprising that numerous human pathogens from diverse genera have developed complement-evasion strategies (23-26).

SA employs various immune evasion strategies: Protein A and Map can interfere with antibody and memory T cell responses, respectively, and super antigens (bacterial toxins) can nonspecifically activate up to 20% of naive T cells resulting in the release of large quantities of cytokines and generating a clinical condition resembling septic shock (1, 18).

This work describes another SA MIM (SAC3) that binds to C3 and interferes with complement activation. Although SAC3 had been previously characterized as a Fgn-binding protein (Efb) (31, 32), it is not unusual for bacterial proteins to have multiple biological activities (e.g. SA Map and Streptococcal M protein) (15-18, 21, 22) and may be one explanation for the capacity of these bacteria to colonize, survive and persist in vastly different environments (e.g. skin, blood, kidney, or bone) and cause such a broad range of diseases.

A large number of human pathogens have evolved mechanisms of complement evasion (23, 42, 43). However, the human pathogens that generate specific C3-binding

proteins is limited to a relatively small group that includes *Trypanosoma cruzi*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Leishmania major*, *Legionella pneumophila*, *Chlamydia trachomatis* and herpes simplex virus (22, 44-51). What role each of these proteins plays in bacterial survival or evasion of host defenses is not completely understood. *M. leprae*, *L. major*, and *L. pneumophila* use C3 as a ligand to facilitate their internalization into mononuclear cells via CR1 and CR3 complement receptors (44, 49, 50). What role the C3-binding proteins from *C. trachomatis* or *M. tuberculosis* play has not been defined clearly, although published reports do not suggest an immunomodulatory function for these proteins (47, 52). *T. cruzi*, *S. pneumoniae*, *P. aeruginosa* and herpes simplex virus type 1 and type 2 can all generate C3-binding proteins with complement inhibitory properties (45, 48, 53-56). It is suggested that the surface glycoprotein C from herpes simplex viruses inhibits alternative pathway activation via blocking the binding of properdin to C3b of the C3 convertase (53-55) and the shed *T. cruzi* CRPs (complement regulatory proteins) can inhibit alternative complement activation by molecular mimicry of the mammalian DAF (decay accelerating factor) (48, 57). *P. aeruginosa* secretes a C3 protease which can inactivate C3 by cleaving the α-chain (51), and the CbpA protein (choline-binding protein) from *S. pneumoniae* likely plays an anti-opsonization/phagocytosis role by binding to C3 (45).

SAC3 represents the first complement regulatory MIM identified from SA and in conjunction with other SA immune evasion mechanisms may further explain the capacity of SA to cause persistent infections. It is interesting that SA and *P aeruginosa* are the only bacteria capable of generating alternative and classical pathway-inhibitor proteins

since both are opportunistic pathogens capable of causing many different disease manifestations.

Although the mechanism by which SAC3 inhibits complement activity is not yet known, it is likely to function by sterically hindering the activation of C3 by preventing its binding to activator surfaces (alternative pathway) or C3 convertase (classical pathway) since C3 incubated in the presence of SAC3 for 1h at 37°C did not result in any discernable degradation products following examination by SDS-PAGE (data not shown). Previous work demonstrated that SA capsular proteins and cell-wall peptidoglycans can provide activator surfaces that can activate the alternative pathway (58, 59), suggesting that complement regulatory proteins like SAC3 may play an important role in SA survival by diminishing or preventing complement activation. That SAC3 is highly conserved among and expressed on all SA strains examined suggests that this protein is critical to SA survival (60).

To our knowledge, SAC3 is the first SA MIM with complement inhibitory properties and therefore represents a novel member of SA's growing immunomodulatory arsenal. A more clear understanding of SAC3, including the elucidation of its tertiary structure, mechanism of action and identification of additional functions may lead to the design of treatments or preventative strategies used to combat SA infections.

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Figure Legends

Fig. 1. SA secretes a C3b-binding protein. Lane 1, *E. coli*; lane 2, *S. carnosus*; lane 3, *S. aureus*; and lane 4, *S. aureus* supernatant. Bacteria or supernatant were fractionated by SDS-PAGE (*a*) or transferred onto a PVDF membrane (*b*). After blocking additional protein-binding sites, proteins on the membranes were probed with digoxigenin-labeled C3b followed by a secondary incubation with AP-labeled anti-digoxigenin Fab fragments and then developed. Membranes probed with secondary antibody alone did not exhibit nonspecific binding (data not shown).

Fig. 2. Labeled C3b and Fgn bind to recombinant SAC3. Lane 1, SAC3; lane 2, SA1755; lane 3, Map19; and lane 4, SdrG. 4 μg of each recombinant protein was subjected to SDS-PAGE (*a*) or transferred onto PVDF membranes. After blocking additional protein-binding sites, proteins on the membranes were probed with either digoxigenin-labeled C3b (*b*) or Fgn (*c*) followed by a secondary incubation with AP-labeled anti-digoxigenin Fab fragments and then developed. Membranes probed with secondary antibody alone did not exhibit nonspecific binding (data not shown).

Fig. 3. SAC3 binds to membrane-bound C3b. Lane 1, C3b; lane 2, human Fgn; lane 3, human serum. Proteins and serum were subjected to SDS-PAGE (*a*) or transferred onto PVDF membranes (*b-c*). After blocking additional protein-binding sites, proteins on the membranes were probed with either SAC3 (*b*) or biotin-labeled chicken anti-human C3 antibodies (c) followed by a secondary incubation with either mouse anti-His antibodies (*b*) or with avidin-AP (*c*). Following a third incubation with anti-mouse AP-conjugated

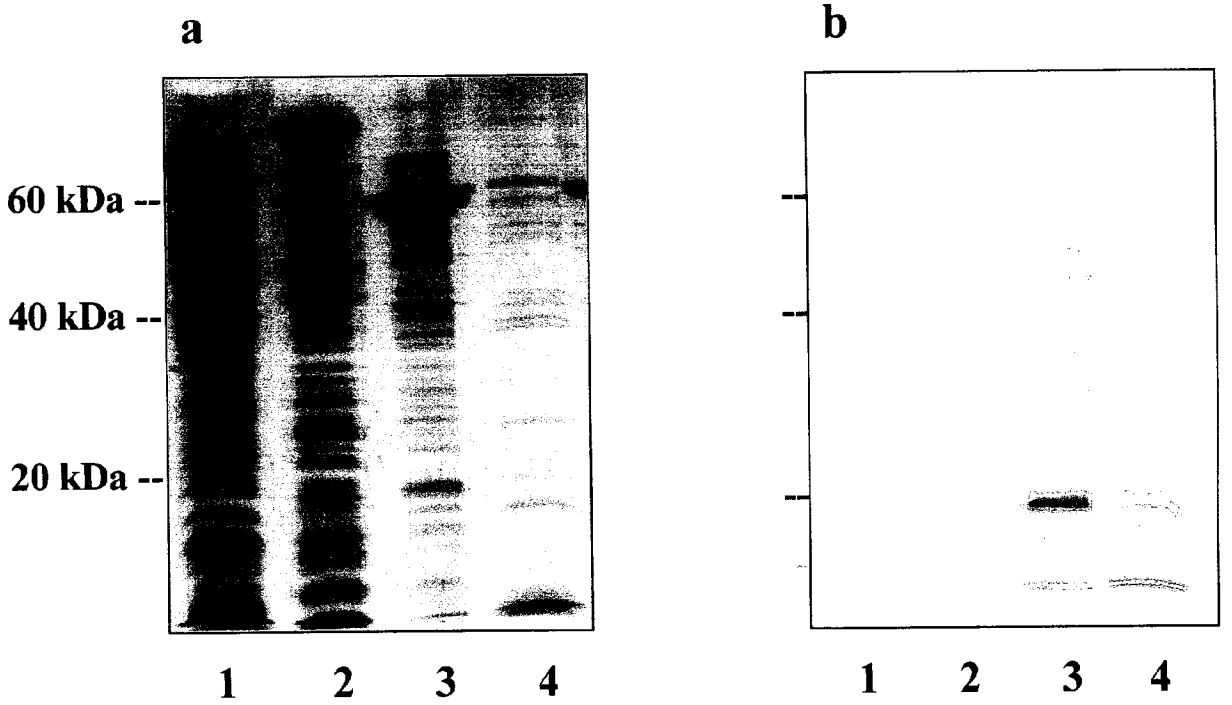
antibodies (*b*) the blots were developed. Neither secondary AP-conjugated secondary antibodies alone nor avidin-AP alone bound nonspecifically to the membranes (data not shown).

Fig. 4. SAC3 binding to C3b- and Fgn-coated microtiter wells. SAC3 or SdrG were used to probe C3b- or Fgn-coated microtiter wells (*a* and *b*, respectively). Binding was detected using a primary monoclonal mouse anti-His followed by a secondary incubation with a goat anti-mouse AP-conjugated antibody. The data are expressed as the mean absorbance (405 nm) ± SE of the mean of triplicate samples.

Fig. 5. SAC3 inhibits complement activation. The effects of SAC3 on complement activation were examined using different assays measuring the classical (*a*) or alternative (*b*) complement activation pathways. Preincubation of human serum with SAC3 but not with control proteins prevented the complement-mediated lysis of erythrocytes. These experiments were performed 3 times and the data are representative of all results.

Abstract

Staphylococcus aureus (SA) is a commensal organism associated with the normal flora of the skin, however, damage to the skin or if other natural barriers are damaged by trauma, inoculation by needles, or direct implantation of medical devices, these organisms can gain entry into the host and cause a variety of diseases or potentially lethal infections. SA can modulate host immunity by a variety of mechanisms that may result in persistent infections. We report here that SA secretes a 19 kDa protein (SAC3) that can bind to the α-chain of the complement protein C3. Amino acid N-terminal sequencing of SAC3 demonstrated a homology to the SA extracellular fibrinogen-binding (Efb) protein suggesting that SAC3 may play multiple roles during the infection process. Western-ligand blot analysis suggested that recombinant SAC3 recognizes a linear epitope in C3b since intense binding was seen to membrane-bound C3b in comparison to fibrinogen, however, direct binding ELISAs demonstrated that SAC3 recognized both plate-bound C3b and fibrinogen in a similar fashion. Recombinant SAC3 exhibited saturation kinetics and bound C3b with an apparent K_D of 0.6×10^{-7} μM. Furthermore, we demonstrate that SAC3 interfered with *in vitro* functional assays that tested both the classical and alternative complement pathways. These experiments suggested that SAC prevents complement activation by binding to and preventing complement activation beyond C3. These data suggest that SAC3 may be a virulence factor that is involved in facilitating persistent *S. aureus* infections by interfering with complement activation *in vivo*.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2a

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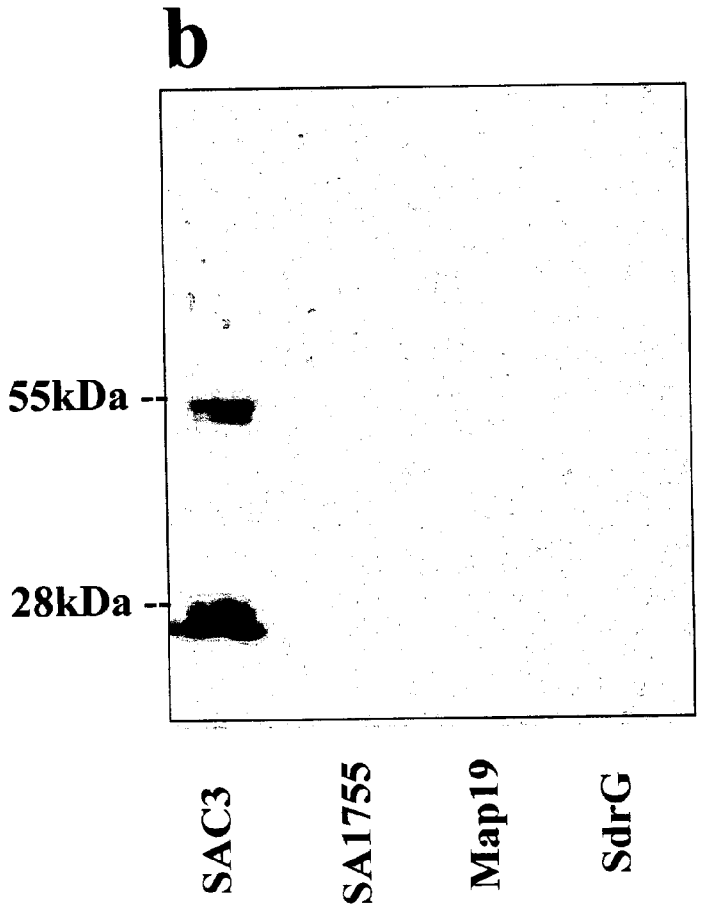

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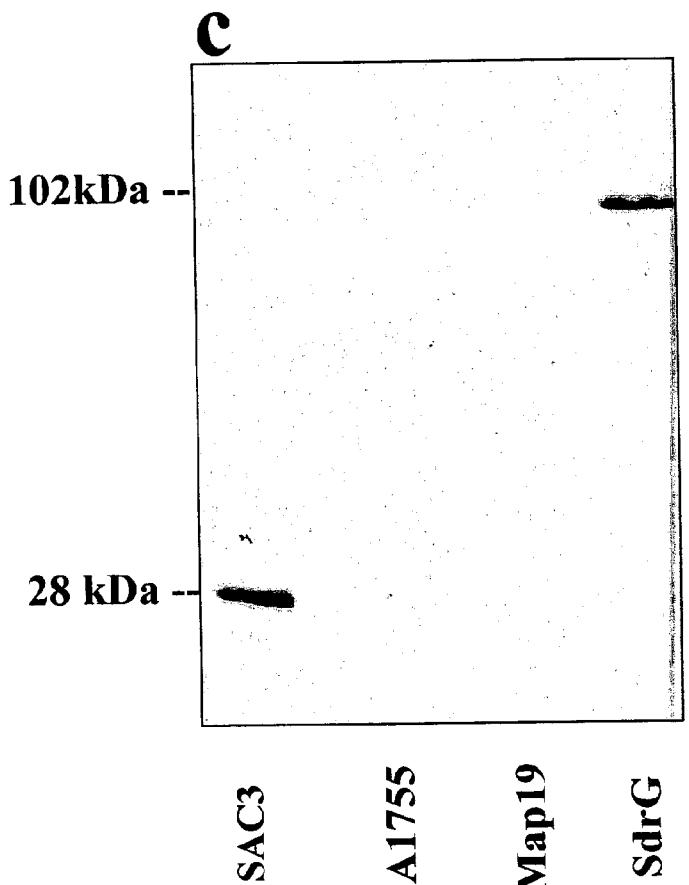

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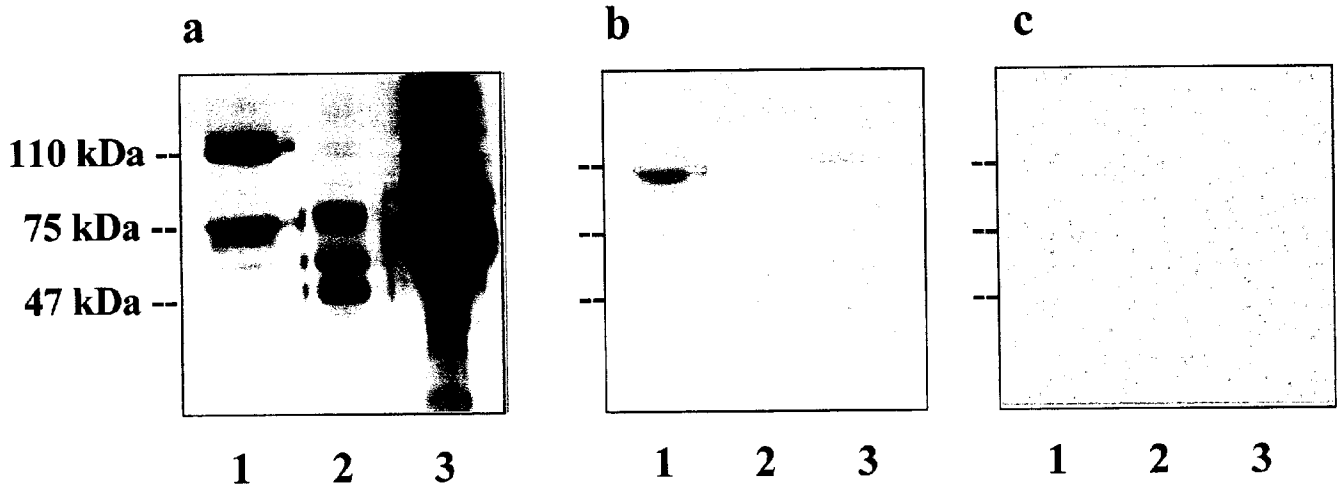

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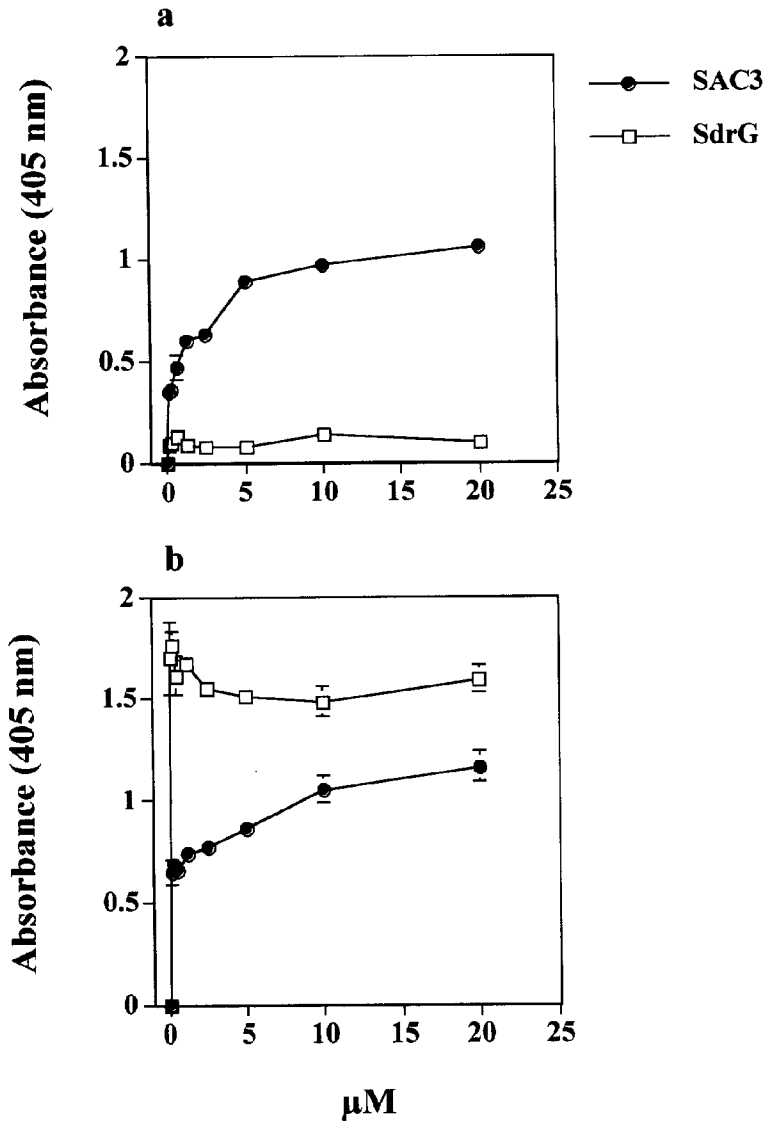
a
b
SAC3
SdrG
Absorbance (405 nm)
Absorbance (405 nm)
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1
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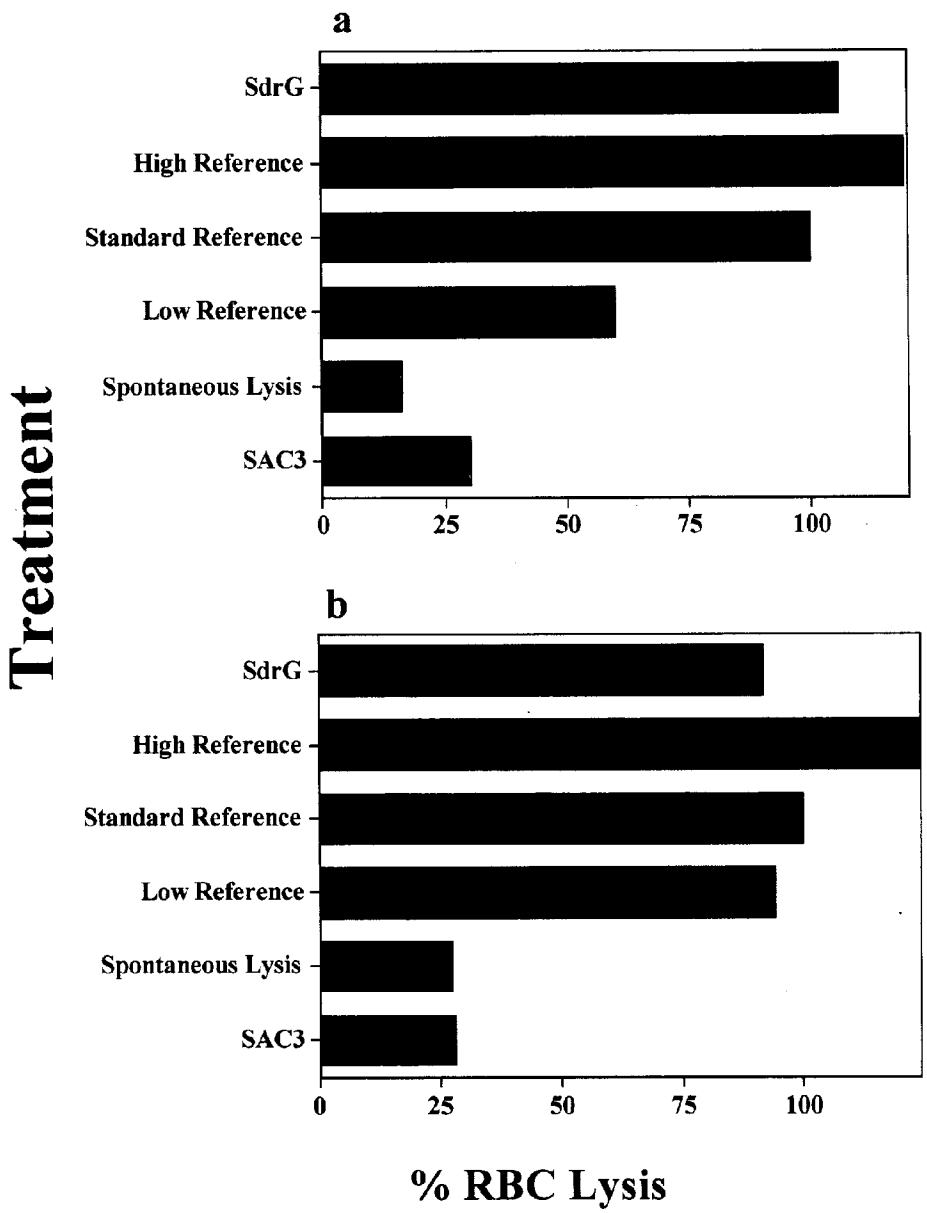
2
0
5
10
15
20
25
μM

5


a
SdrG
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